Preparation and evaluation of combined vaccine against necrotic enteritis and colibacillosis in chickens and detection of maternal immunity in their progeny

Noura M. Khalaf; Hazem M. Ibrahim [1]; Wafaa R. Abdel-Aziz[1]; Alaa A. Elmenisy[1]; Rafik H. Sayed[2]; Lamees A. El-Tantawy[1] and Fady A. Shasha[2]

[1]*Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt*

[2]*Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt.*

A B S T R A C T

Necrotic enteritis (NE) caused by *Clostridium perfringens* type A and colibacillosis caused by Avian pathogenic *E. coli* (APEC), are two pathogenic diseases that threaten the poultry industry worldwide. A combined inactivated vaccine from *Clostridium perfringens* type A toxoid and serotypes O_1 and O_{78} of E-coli adjvunated with montanide gel was prepared and evaluated in two weeks old SPF white Lohman layer chickens and its progeny. The prepared vaccine was found safe and produced antitoxic titre against NE of 10 IU after 22 week of vaccination as measured by serum neutralization test and 2692 ELISA titre. Also it produced a humeral antibody titre against E.coli serotypes used of 80 at the 22^{th} week post vaccination by microagglutination test (MAT) and an 80% protection in challenge against virulent E.coli serotypes used.

Conclusion: vaccination of chicken with two doses, 3 weeks apart, of combined vaccine of *Clostridium perfringens* type A toxoid and serotypes O_1 and O_{78} of E-coli adjvunated with montanide gel, could protect against necrotic enteritis and colibacillosis.

Keywords: Necrotic enteritis- Colibacillosis - *Clostridium perfringens*-vaccine-chicken

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1. INTRODUCTION

Necrotic enteritis (NE) is a disease of broilers and hens either breeders or layers at different ages starting from two weeks until twelve weeks old.

Clostridium perfringens type A is the most common cause of poultry NE because it produces alpha toxin which has long been considered as a major factor in pathogenesis of NE (Cooper *et al.*, 2010).

Avian pathogenic E.*coli (*APEC) is one of the most important opportunist pathogens in industrialized poultry production. APEC is associated with a variety of extra intestinal disease syndromes. In young chicks, APEC-induced omphalitis (yolk sac infection) which may result in generalized septicemia with perihepatitis, whereas respiratory infection may occur after aerogenic transmission (Pires dos Santos *et al.*, 2013).

Colisepticemia usually occurs among birds with 2 to 12 weeks of age, with the majority of the cases occurring among birds

with 4 to 9 weeks of age with mortality reaching rates as high as 20% (Dho-Moulin and Fairbrother, 1999).

A vaccine for NE of chicken would reduce the need to prevent or treat the disease in broiler chickens with antibacterial drugs (Thompson *et al.,* 2006).

With increased regulation on use of antibiotics to prevent infections and antibiotic resistance in bacteria, especially E.coli, alternatives are needed to improve animal health and welfare, such as new vaccines (Mellata, 2013).

One day old chicks has ill developed immune system depending mainly on maternal derived immunity and become sensitive to different pathogens, moreover this ill developed immune system resulting in weak antibody response to vaccinal program, this problem affects mass parenteral vaccination at day one which need enhancing immune response as it is not applicable (Mot *et al.*,2014).

Controlling of these diseases by active immunization is of considerable importance for better control over the disease spread and ultimately eradication of NE & Colibacillosis diseases in chickens with minimum cost as combined vaccines have the advantage of protecting against more than one disease at the same time, beside, reducing vaccination expenses, number of vaccination performed and saving time.

So the aim of the present work was to prepare a combined inactivated vaccine from *Clostridium perfringens* type A toxoid and serotypes O1 and O78 of E-coli adjvunated with montanide gel for improving its immunogenicity. Also, evaluating the protective efficacy of the prepared vaccines by SNT and ELISA for *Clostridium perfringens* type A and microagglutination test, challenge test and ELISA for E-coli. The study also monitors the levels of antibodies in newly hatched chicks during the first three days of age.

2.MATERIAL AND METHODS

2.1. Bacterial Strains:

2.1.1. Clostridium perfringens type A strain:

A locally isolated strain obtained from Anaerobic Vaccines Research Department, Veterinary Serum Vaccine Research Institute, Abbasia, Cairo, Egypt, was used for vaccine preparation.

2.1.2. E coli strains:

Two *E.coli* strains serotypes O_1 and O_{78} were kindly obtained from Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo, Egypt, were used for vaccine preparation.

2.2. Laboratory animals:

2.2.1. SPF one day old chicks:

Thirty chicks were used for safety testing of the prepared vaccines (10 chicks for each vaccine).

2.2.2. SPF white Lohman layer chickens:

A total number of 160, two weeks old SPF white Lohman layer chickens were obtained from SPF farm at Koom Osheem Fayuom Province, Egypt. They were housed in batteries with the network floor. All birds were ascertained first to be free from *Clostridium perfringens* type A and E.coli serotypes O_1 and O_{78}(organism and antibodies). They were fed on free balanced ration, and used for evaluation of prepared vaccines.

2.2.3. Mice:

A total number of 100 swiss mice, were used for determination of safety tests for the prepared vaccines and serum neutralization test (SNT).

2.3. Vaccine preparation:

2.3.1.Necrotic enteritis vaccine

Clostridium perfringens type A toxoid was prepared according to Ahmed (1975). 0.5 ml of vaccine (equal to 1 vaccinal dose) was adjusted to contain 100 MLD of *C.*

perfringens alpha toxoid. Then montanide gel was added (SEPPIC®, France) in a ratio of 20 adjuvant: 80 antigen.

2.3.2. Colibacillosis vaccine: was prepared according to Chaffer et al., (1997)

E.coli strains serotypes O_1 and O_{78} were grown separately onto brain heart agar in Roux bottles and incubated at 37ºC for 24 hr. The colonies were collected using normal saline then mixed together and bacterial suspension was adjusted to be $1x10^9$ CFU/0.5 ml (vaccinal dose). The bacteria were then inactivated by adding 0.5% formalin with agitation then montanide gel (SEPPIC®, France) was mixed with bacterial suspension in a ratio of 20 adjuvant: 80 antigen.

2.3.3. Combined vaccine against necrotic enteritis and Colibacillosis:

A combined vaccine from *Clostridium perfringens* type A toxoid (100 MLD of *C. perfringens* alpha toxoid/dose) and inactivated *E.coli* strains serotypes O_1 and O_{78} ($1x10^9$ CFU/dose) adjuvanted with montanide gel (SEPPIC®, France) was prepared; the vaccinal dose was adjusted to be 0.5 ml.

All vaccines were subjected to safety and sterility test before immunization according to British Pharmacopeia (2010) and OIE (2017).

2.4. Experimental design:

Chickens were divided into 4 groups, 40 for each group as follow:

Group (1): was vaccinated with necrotic enteritis vaccine.

Group (2): was vaccinated with Colibacillosis vaccine.

Group (3): was vaccinated with combined vaccine.

Group (4): was left as control (inoculated with 0.5 ml saline).

Each chicken in first, second and third group was inoculated with 0.5 ml of each vaccine S/C in the middle dorsal back of neck two doses with 3 weeks interval for each vaccine. Serum samples were collected regularly before immunization, 2, 3, 6, 10, 14, 18, 22 weeks after 1st vaccination. Sera of each group were pooled and kept at -20^0C until used for evaluating the developed humoral immune response.

-Hatched chicks were collected from eggs of vaccinated hens at 22 weeks of age. Then they were bled for serum collection at day one and day three.

2.5. Quality control testing of the prepared experimental vaccines:

2.5.1. Sterility test:

The prepared vaccines were tested to be free from any contaminant (aerobic and anaerobic bacteria, fungus and mycoplasma according to OIE (2017).

2.5.2. Safety test:

Safety of the prepared vaccines was tested according to OIE (2017); 10 chicks one day old were injected subcutaneously with double field dose of the prepared vaccines. The inoculated chicks were observed for 14 successive days to detect any signs of local or systemic reaction.

2.5.3. Determination of immune response against the prepared vaccines:

2.5.3.1. Serological evaluation of humeral immune response of the vaccinated chickens against Clostridium perfringens type A:

2.5.3.1.1. Antitoxin assay by SNT:

Pooled sera of each group were tested for determination of the alpha antitoxin titer of *Clostridium Perfringens* type A using SNT in mice according to Gadalla *et al*. (1971)

2.5.3.1.2. Antitoxin assay by ELISA:

ELISA was performed on the serum sample, according to the method described by Voller *et al*. (1976) and Briggs and Skeels (1984). The results were calculated according to the following formula:

S/p (sample/positive)

$$= \frac{\text{sample mean} - \text{negative control}}{\text{positive control} - \text{negative control}}$$

Log_{10} titre= 1.08(log_{10} S/P) + 3.82

Titre = Anti-log_{10}

2.5.3.2. Serological evaluation of humeral immune response of the vaccinated chickens against E coli serotypes O_1 and O_{78}:

2.5.3.2.1. Micro-agglutination test (MAT):

Antibody response in vaccinated and unvaccinated chickens was followed up on regular intervals post vaccination determined by Micro-agglutination test (MAT) using sonicated antigen, according to the method described by Thaxton *et al.* (1970) and Brown *et al.* (1981)

2.5.3.2.2. ELISA:

ELISA was performed on the same serum sample, according to the method described by Voller *et al.* (1976) and Briggs and Skeels (1984). The results were calculated according to the following formula:

S/p (sample/positive)

$$= \frac{\text{sample mean} - \text{negative control}}{\text{positive control} - \text{negative control}}$$

Log_{10} titre= 1.09(log_{10} S/P) + 3.63

Titre = Antilog_{10}

2.5.4. Challenge test:

Three weeks post-boostering, group 2 and 3 were subdivided into two subgroups; First subgroup were injected into the thigh region with 0.2 ml containing 10^7 CFU of E.coli serotype O_1 and the second subgroup were injected with E.coli serotype O_{78} and monitored for clinical signs, Mortality was recorded for 7 days after challenge according to the method described by Chaffer *et al.* (1997).

3. RESULTS

The prepared vaccines were found to be safe and sterile during the period of observation.

The results of SNT table (1) showed that the antitoxin titer of chickens vaccinated with *Clostridium perfringens* type A only showed increasing antibody titer begins with 15 IU 2 weeks after vaccination, reached peak (25 IU) at the 10th week post vaccination, then decreased and reached 10 IU after 22 weeks. While the antitoxin titer of chickens vaccinated with combined vaccine begins with 10 IU 2 weeks post vaccination, then reached the peak (20 IU) 10 weeks post vaccination and declined till reaching 10 IU at the 22th week post vaccination.

ELISA titers in table (2) against *Clostridium perfringens* type A alpha toxin in serum of vaccinated chickens came in parallel with the SNT results.

The humeral immune response against E.coli serotypes O_1 and O_{78} was measured by MAT as illustrated in tables (3, 4). It was clear that for both antigens the titer start with 40 at 2nd week post first vaccination then increased and reached peak at 6th week to 320 then start decreasing at 18th week and reached 80 at 22th week for both vaccines.

Also the humeral immune response against E. coli serotypes O_1 and O_{78} was measured by ELISA as shown in tables (5, 6). For O_1 antigen the titer start with 850 and 870 at 2nd week post first vaccination then increased and reached peak at 10th week to 4345 and 4593 then start decreasing at 18th week and reached 3479 and 3550 at 22th week for E-coli vaccine and combined vaccine, respectively.

On the other hand, for O_{78} antigen the titer starts with 920 and 942 at 2nd week post first vaccination then increased and reached peak at 10th week to 4371 and 4403 then start decreasing at 18th week and reached 3264 and 3378 at 22th week for E-coli vaccine and combined vaccine, respectively.

The protection rate in table (7) measured by challenge test was 80% for both vaccines (*E. coli* vaccine & combined vaccine) in chickens.

Table (8) showed the SNT and ELISA titers of hatched chickens from vaccinated hen with combined vaccine. The SNT titer raised

from1U in day one to 1.5 U in day three, also ELISA titer increased from 1413 to 2455 in day three. Regarding antibody titers against E-Coli $O_1$and E-Coli O_{78}, it was 40 U by MAT for both serotypes. While by ELISA there was a slight difference between the two serotypes.

Table (1): Mean antitoxin titer against *Clostridium perfringens* type A alpha toxin in serum of vaccinated chickens as measured by SNT

Groups	Mean antitoxin Titre (expressed as IU)							
	Prevacc.	Weeks post vaccination						
		2	3	6	10	14	18	22
Group (1)	0	15	15	20	25	15	15	10
Group (3)	0	10	15	15	20	15	10	10
Group (4)	0	0	0	0	0	0	0	0

Group (1): chickens vaccinated with necrotic enteritis vaccine adjuvanted with montanide gel.

Group (3): chickens vaccinated with inactivated combined vaccine adjuvanted with montanide gel.

Group (4): Control unvaccinated group

Table (2): ELISA titer against alpha toxin of *C. perfringens* in sera of vaccinated chickens

Groups	Mean ELISA Titre							
	Weeks post vaccination							
	Prevacc.	2	3	6	10	14	18	22
Group (1)	150	4467	4677	7413	8318	5623	4732	3767
Group (3)	167	4677	5370	6026	7324	3890	3090	2692
Group (4)	100	166	166	170	187	155	150	100

Table (3): Microagglutination titer in sera of vaccinated chickens against E. coli O_1

Groups	Microagglutination Titre							
	Weeks post vaccination							
	Prevacc.	2	3	6	10	14	18	22
Group (2)	0	40	80	320	640	320	160	80
Group (3)	0	40	80	320	320	160	160	80
Group (4)	0	0	0	0	0	0	0	0

Group (2): chickens vaccinated with inactivated *E. coli* vaccine adjuvant with montanide gel.

Group (3): chickens vaccinated with inactivated combined vaccine adjuvant with montanide gel.

Group (4): Control unvaccinated group

Table (4) Microagglutination titer in sera of vaccinated chickens against E-Coli O_{78}

Groups	Microagglutination Titre							
	Weeks post vaccination							
	Prevacc.	2	3	6	10	14	18	22
Group (2)	0	40	80	320	640	320	320	80
Group (3)	0	40	80	320	320	320	160	80
Group (4)	0	0	0	0	0	0	0	0

Group (2): chickens vaccinated with inactivated *E. coli* vaccine adjuvant with montanide gel.
Group (3): chickens vaccinated with inactivated combined vaccine adjuvant with montanide gel.
Group (4): Control unvaccinated group

Table (5) ELISA titer in sera of vaccinated chickens against E.coli serotype O_1

Groups	ELISA Antibody Titre							
	Weeks post vaccination							
	Prevacc.	2	3	6	10	14	18	22
Group (2)	96	850	2412	4150	4345	4150	3818	3479
Group (3)	117	870	2755	4217	4593	4217	3890	3550
Group (4)	100	120	240	166	188	166	157	100

Group (2): chickens vaccinated with inactivated *E. coli* vaccine adjuvant with montanide gel.
Group (3): chickens vaccinated with inactivated combined vaccine adjuvant with montanide gel.
Group (4): Control unvaccinated group

Table (6) ELISA titer in sera of vaccinated chickens using O_{78} antigen

Groups	ELISA Antibody Titre							
	Weeks post vaccination							
	Prevacc.	2	3	6	10	14	18	22
Group (2)	111	920	2532	3960	4371	4205	3731	3264
Group (3)	123	942	2675	4078	4403	4327	3780	3378
Group (4)	114	125	140	166	173	182	167	130

Group (2): chickens vaccinated with inactivated *E. coli* vaccine adjuvant with montanide gel.
Group (3): chickens vaccinated with inactivated combined vaccine adjuvant with montanide gel.
Group (4): Control unvaccinated group

Table (7): Results of challenge test with E-coli serotypes O_1 and O_{78} among chickens vaccinated with the prepared vaccines

Groups	Total No. of challenged birds	No. of dead birds ⁄ Total birds		Protection rate
		O_1	O_{78}	
Group (2)	20	2⁄10	2⁄10	80%
Group (3)	20	2⁄10	2⁄10	80%
Group (4)	20	8⁄10	2⁄10	20%

Table (8): specific IgG titers in the sera of hatched chicks by SNT and ELISA

Test	Day one old	Day three old	Control
SNT	1 U	1.5 U	0 U
ELISA	1413	2455	631

Table (9): Antibody titers in the sera of hatched chicks by microagglutination test (MAT) and ELISA

Test	Serotypes	Day one old	Day three old	Control
MAT	O_1	40	40	0 U
	O_{78}	40	40	0 U
ELISA	O_1	1775	1805	120
	O_{78}	1690	1710	115

4. DISCUSSION

Necrotic enteritis represents one of the most important diseases that threaten poultry population (Tripathy and Reed, 2008), it causes severe economic losses by increasing mortality rates.

It was well known that vaccination is the cornerstone in controlling infectious diseases and it is known that flocks with high titers of maternal antibodies against alpha toxin had lower mortality during the production period than flocks with low titers (Heier *et al*., 2001).

Inactivated vaccines based on formalin or heat inactivated E. coli are generally believed to confer protection against avian Colibacillosis in an antibody-dependent manner (Arp, 1980 and Leitner *et al*., 1990). Because cross-protection is usually not observed with E. coli serotypes from poultry (Arp, 1980), a suitable vaccine would have to contain the most common serotypes.

So, the present work was to prepare a combined inactivated vaccine from *Clostridium perfringens* type A toxoid and serotypes O_1 and O_{78} of E-coli adjvunated with montanide gel for improving its immunogenicity. The study also evaluated the protective efficacy of the prepared vaccines by challenge test and evaluation of the immunizing and protective values of the prepared vaccines by SNT and ELISA For *Clostridium perfringens* type A and microagglutination test and ELISA for E.coli.

The results of SNT showed that the antitoxin titer of chickens vaccinated with two doses of *Clostridium perfringens* type A toxoid only showed increasing antibody titer begins with 15 IU and reached 10 IU after 22 weeks. While the antitoxin titer of chickens vaccinated with combined vaccine begins with 10 IU and reached the peak (20 IU) then declined to 10IUat the 22th week post vaccination. The protective antibody titer against the alpha toxin of *Clostridium perfringens* is reported to be 0.5 IU/ml for alpha toxin (British Pharmacopeia, 2010). As egg production starts approximately at the 16-18 week of age in chicken, the antibody titer present is sufficient to confer immunity.

ELISA procedure is a rapid, specific and sensitive serological assay which has been used for the detection of several bacterial toxins.ELISA results come in parallel with the results of SNT. These results agreed with Heier *et al* (2001) and Lovland *et al* (2004), who showed that the vaccination of chickens with NE vaccine resulted in strong and specific antibody against alpha toxin of *Clostridium perfringens* type A.

Several authors have reported the isolation of E.coli strains O_1 and O_{78} as one of the prevalent serotypes in Egypt as Abd El Tawab *et al.* (2016), Younis *et al.* (2017) and Moawad *et al.* (2018). so, these two strains were selected. The humeral immune response against E.coli serotypes O_1 and O_{78} was measured by MAT and ELISA as illustrated in tables 3,4. it was clear that for both antigens the titer start rising at 2nd week post first vaccination then increased and reached peak at 6th week to 320 then start decreasing at 18th week to reach 80 by the 22th week for both vaccines.

Also the humeral immune response against E. coli serotypes O_1 and O_{78} was measured by ELISA as shown in tables 5and 6.

For O_1 antigen the titer start with 850 and 870 at 2nd week post first vaccination then increased and reached peak at 10th week to 4345 and 4593 then start decreasing at 18th week and reached 3479 and 3550 at 22th week for E-coli vaccine and combined vaccine, respectively. On the other hand, for O_{78} antigen the titer starts with 920 and 942 at 2nd week post first vaccination then increased and reached peak at 10th week to 4371 and 4403 then start decreasing at 18th week and reached 3264 and 3378 at 22th week for E-coli vaccine and combined vaccine, respectively. These results agree with that obtained by Sadeyen *et al.* (2015) and El Jakee *et al*. (2016) who found that inactivated APEC vaccines are protective against experimental intra-air sac challenge in a turkey model of acute colibacillosis and that they both induce predominantly a Th2 response in the spleen that correlates with elevated APEC-specific antibody levels.

The protection rate measured by challenge test was 80% for both vaccines (*E. coli* vaccine & combined vaccine) in chickens and these results agreed with El Jakee *et al*. (2016).

Maternal antibody were detected in hatched chicks, as shown in table (8 and 9), the chicks have protective levels of both antigens in day one and day three. These results come as Lovland *et al.* (2004) who reported a strong serum immunoglobulin G response to *C. perfringens* alpha-toxin in parent hens, and specific antibodies were transferred to their progeny. Also, these results are in agreement with Heller *et al.* (1990) who stated that at hatching, the level of maternal antibody to *E. coli* in chicks, measured by ELISA was found to be 55 to 62% of that of the hen. It declined to an undetected level at 21 days of age.

From results obtained in this study it could be concluded that vaccination of chicken with combined vaccine of E coli and *Clostridium perfringens* type A vaccine is recommendable to be used for better controlling and eradication of necrotic enteritis and colibacillosis in chickens.

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